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Immunology

Development of B-lineage cells in the bone marrow of *scid/scid* mice following the introduction of functionally rearranged immunoglobulin transgenes

(severe combined immunodeficiency/μ-transgenic scid mice/$\mu\kappa$-transgenic scid mice/pre-B cells/fluorescence-activated cell sorter)

MICHAL REICHMAN-FRIED, RICHARD R. HARDY, AND MELVIN J. BOSMA

Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111

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ABSTRACT **Mice homozygous for the mutation *scid* (scid mice) are severely immunodeficient and generally lack detectable numbers of pre-B, B, and T cells. This condition is believed to result from a defect in the mechanism responsible for rearrangement of immunoglobulin and T-cell receptor genes in developing B and T lymphocytes. To test this hypothesis and evaluate whether *scid* affects only the process of gene recombination, we introduced functionally rearranged immunoglobulin genes into the scid mouse genome. As scid mice appear to contain early lymphoid cells committed to the B lineage (pro-B cells), we asked whether the introduction of an IgM heavy-chain gene alone (μ-transgenic scid mice) or both IgM heavy- and κ light-chain genes ($\mu\kappa$-transgenic scid mice) would allow further differentiation of scid pro-B cells into pre-B and B cells. We found that normal numbers of pre-B cells appeared in the bone marrow of μ-transgenic scid mice and that both pre-B and B cells appeared in the bone marrow of $\mu\kappa$-transgenic scid mice. However, in the latter case, the number of pre-B and B cells was 2- to 3-fold less than in the controls ($\mu\kappa$-transgenic scid heterozygotes) and few, if any, B cells were detectable in the peripheral lymphoid tissues. The implications of these results for the above hypothesis are discussed.**

Mutant C.B-17/Icr *scid/scid* mice with severe combined immunodeficiency (1), here referred to as scid mice, are homozygous for an autosomal recessive mutation (*scid*) on chromosome 16 (2). Although scid mice show evidence of early lymphoid cells committed to the B- or T-cell pathway (3), these cells generally fail to mature into detectable numbers of pre-B, B, and T cells (1, 4). It has been hypothesized (5) that developing scid lymphocytes die prematurely as a result of a defective lymphocyte recombinase system, which cannot successfully recombine the variable (V), diversity (D), and joining (J) gene elements that code for immunoglobulin (Ig) and T-cell receptor variable regions. This hypothesis was prompted by the observation of abnormal Ig heavy-chain and T-cell receptor gene rearrangements in transformed lymphoid cell lines recovered from scid bone marrow or thymus (5). Others have confirmed and extended these findings (6–8); moreover, abnormal Ig gene rearrangements also have been observed in long-term cultures of scid bone marrow cells (9, 10). Further support for this hypothesis has come from reports showing that transformed scid lymphocytes have an abnormal VDJ recombinase activity (11, 12). Though this activity can mediate the joining of signal sequences that flank V, D, and J coding elements and thus yield the reciprocal product of a standard VDJ recombination event, it cannot mediate with any appreciable frequency the functional joining of V(D)J elements. Failure to recombine chromosome ends bearing V, D, or J coding elements presumably would be lethal to developing scid lymphocytes.

To test the above hypothesis, we introduced functionally rearranged Ig transgenes [i.e., a μ heavy-chain gene alone or both a μ gene and κ light-chain gene ($\mu\kappa$)] into the scid mouse genome. In normal developing B-lineage cells, the μ locus rearranges before the κ locus and cells progress from the μ-expressing pre-B-cell stage to B cells that express both μ and κ chains and are surface IgM$^+$ (reviewed in ref. 13). As neither cell type is detectable in scid bone marrow, it was important to know whether the introduction of μ and $\mu\kappa$ transgenes into the scid mouse genome would result in a stepwise appearance of pre-B cells and B cells, respectively. Such a result would be expected if (*i*) the effect of *scid* on developing lymphocytes were restricted to VDJ recombination and the products of the μ and $\mu\kappa$ transgenes were able to provide the necessary signals for further B-cell maturation, and (*ii*) the Ig transgenes were able to exclude or inhibit rearrangement of endogenous Ig alleles (allelic exclusion) and thereby rescue developing scid lymphocytes from the presumed deleterious effects of endogenous Ig gene rearrangements. We found that the bone marrow of μ-transgenic scid mice contained normal numbers of pre-B cells but no detectable B cells, whereas in the bone marrow of $\mu\kappa$-transgenic scid mice, both pre-B and B cells were detected. But in the latter case, the number of detectable pre-B and B cells was 2- to 3-fold less than in the controls ($\mu\kappa$-transgenic scid heterozygotes); moreover, few, if any, B cells were detectable in the peripheral lymphoid tissues of $\mu\kappa$-transgenic scid mice. We discuss the possibility that incomplete allelic exclusion of endogenous scid Ig alleles may be responsible for the relatively few B cells in $\mu\kappa$-transgenic scid mice.

MATERIALS AND METHODS

Mice. The mutation *scid* occurred in the C.B-17/Icr (C.B-17) inbred mouse strain, an *Igh* allotype congenic partner strain of BALB/c AnIcr (BALB/c) (1). Mice homozygous for *scid* are here designated as scid mice. Mice of the M54 transgenic line carry a functional μ heavy-chain transgene, as described by Grosschedl *et al.* (14), and those of the 207-4 transgenic line carry both a μ and a κ ($\mu\kappa$) transgene, as described by Storb *et al.* (15). The constructed mouse stock homozygous for the SJL allele of the Ig λ light-chain locus (*Igl-1*^{b}) was derived from the work of Epstein *et al.* (16).

Western Blot Analysis. Preparation of cell lysates, electrophoresis, and Western blotting were performed as described (17). The blots were first sequentially overlaid with affinity-purified goat anti-mouse IgM (15 μg/ml) (Fisher) and ^{125}I-labeled IgM-λ (MOPC-104E) to reveal μ chains. A second sequential overlay of affinity-purified goat anti-mouse Ig κ (15

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Abbreviations: FACS, fluorescence-activated cell sorter; V, variable; D, diversity; J, joining.

μg/ml) (Fisher) and ^{125}I-labeled IgG-κ (MOPC 31-C) was carried out to detect κ chains. Proteins were radiolabeled with ^{125}I (Amersham) by using Iodo-Gen (Pierce) as detailed in ref. 18.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Single-cell suspensions were prepared from lymphoid tissues and examined by multiparameter FACS analysis using a dual laser dye laser FACStar^{PLUS} (Becton Dickinson) equipped with filters for four-color analysis (triple immunofluorescence plus propidium iodide staining). Cells were stained as described (19) and 30,000 or 100,000 cells were examined per analysis. Plots present cells falling within forward and large-angle light-scatter gates set to exclude nonlymphoid cells and debris. Data are presented as 5% probability contour plots.

RESULTS

Construction of μ- and μκ-Transgenic scid Mice. The introduction of functionally rearranged μ and μκ gene constructs (transgenes) into the scid mouse genome was accomplished by means of selective genetic crosses with two previously established transgenic mouse lines. This is detailed and illustrated in Fig. 1.

Western Blot Analysis of μ- and μκ-Transgenic scid Mice. To ascertain whether μ and κ chains were synthesized in μ- and μκ-transgenic scid mice, lysates of their lymphoid tissues along with those of littermate controls were examined by Western blot analysis. As shown in Fig. 2*A*, μ chains but not κ chains were present in the bone marrow, spleen, and thymus of μ-transgenic scid mice. No μ chains were detected in nontransgenic scid mice. Since the synthesis of transgene-encoded μ chains in thymocytes is a property of Ig-transgenic mice (14, 15), the detection of a novel-size (78-kDa) μ chain in thymus and bone marrow of μ-transgenic scid mice indicates that the 78-kDa μ chain corresponds to the transgene product. With respect to κ chains, both transgenic and endogenous κ chains were present in the bone marrow of μκ-transgenic heterozygous scid mice, whereas transgenic κ chains alone (together with μ chains) were seen in the bone marrow of μκ-transgenic scid mice (Fig. 2*B*). The κ transgene product can be distinguished from most other κ light chains due to its slower migration (21). Relatively high levels of endogenous κ chains were present in the spleen of μκ-transgenic scid heterozygotes (Fig. 2*B*). In contrast, the spleen of μκ-transgenic scid mice contained relatively low levels of transgenic κ chains and only about 20% of such mice produced detectable levels of serum Ig (0.2–0.5 mg/ml); the majority lacked detectable serum Ig (<0.1 mg/ml) (serological data not shown).

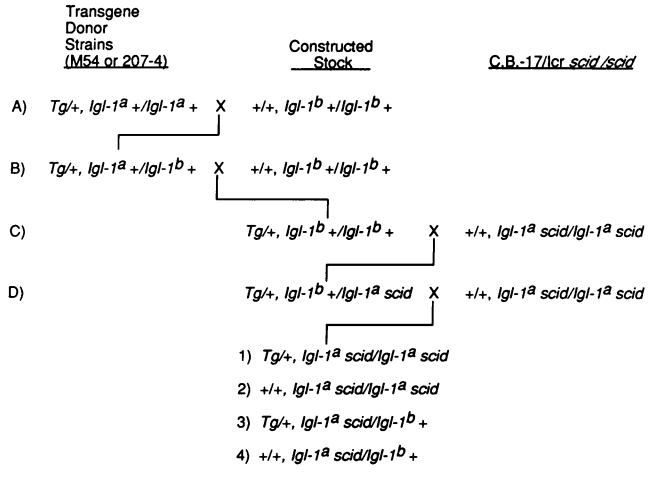

FIG. 1. Construction of transgenic scid mice. The M54 and 207-4 transgenic mouse strains were the genetic donors of the μ and μκ transgenes (*Tg*), respectively. Both genetic donors were heterozygous for their respective *Tg* (*Tg*/+), each integrated in multiple copies at single chromosomal sites and each containing membrane and secretory μ exons (14, 15). The transgenes (*Tg*) were first crossed onto a constructed mouse stock (16) having an Ig light-chain locus (*Igl-1^b*) distinct from the *Igl-1^a* locus of M54, 207-4, and scid mice. Since *Igl-1* is closely linked to *scid* (2) and since *Igl-1^a* and *Igl-1^b* can be distinguished on the basis of a restriction fragment length polymorphism (20), we used *Igl-1^a* as a positive marker for *scid* in the subsequent crossing of the *Tg* onto the scid mouse background (each of the *Tg* segregated independently of *Igl-1*). Four experimental classes of mice were generated by backcrossing transgenic heterozygous scid mice to the scid strain, as indicated in cross D: 1, transgenic scid mice; 2, nontransgenic scid mice; 3, transgenic heterozygous scid mice; and 4, nontransgenic heterozygous scid mice; classes 2, 3, and 4 served as controls for class 1. The class genotypes were identified by dot blot hybridization of tail DNA with a probe (pBR322) specific for prokaryotic vector sequences that flank the μ and μκ transgenes and by Southern blot hybridization of tail DNA with a probe (Cλ1-4E) specific for the *Igl-1* constant region (16). Mice were subsequently tested for the presence of serum Ig to exclude any potential recombinant mice having an *Igl-1^a* + or *Igl-1^b* *scid* chromosome. All mice were used at 2–4 months of age and were obtained from backcross generations 1–5.

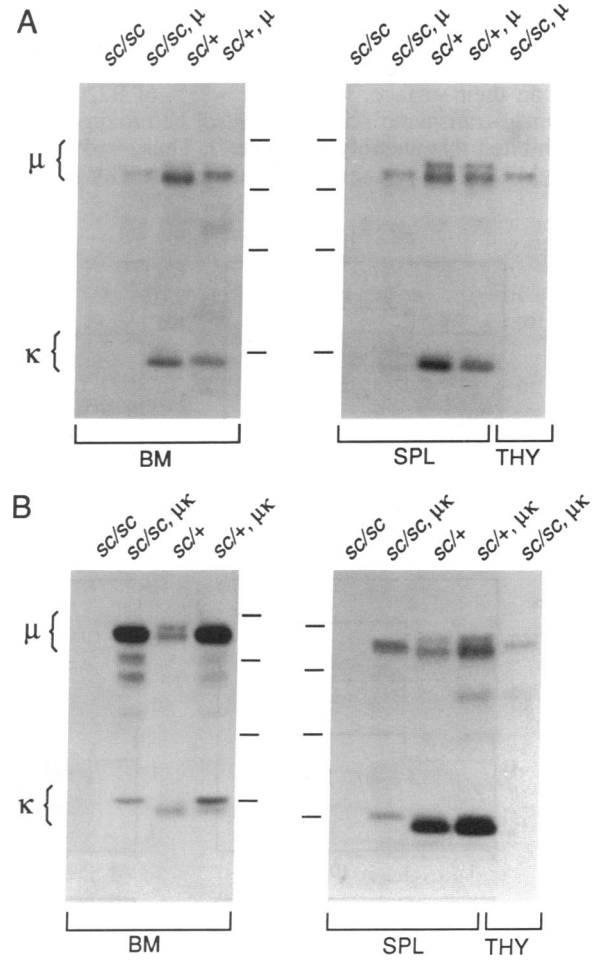

FIG. 2. Evidence of transgene expression in μ- and μκ-transgenic scid mice. Western blot analysis of cell lysates from bone marrow (BM), spleen (SPL), and thymus (THY) of μ-transgenic scid (*sc/sc*, μ) mice (*A*) and μκ-transgenic scid (*sc/sc*, μκ) mice (*B*). Bone marrow and spleen lysates from littermate control mice are included: i.e., *scid/scid* homozygotes (*sc/sc*) and *scid/+* heterozygotes (*sc/+*) with or without the respective transgenes. The positions of μ and κ chains and of the molecular mass standards (103, 67, 42, and 28 kDa) are shown. *B* represents two independent blots with a slight difference in alignment of the molecular mass standards. The 76- and 80-kDa μ chains in *sc/+* and *sc/+*, μ mice may reflect differences in glycosylation. The bands at 50 and 65 kDa represent truncated μ chains.

FACS Analysis of μ- and $\mu\kappa$-Transgenic scid Mice. To evaluate the effect of the μ and $\mu\kappa$ transgenes on the development of B-lineage cells, we analyzed bone marrow and spleen cells of transgenic scid mice and control mice for cell surface expression of B220, Thy-1, and IgM. The B220 antigen is present on both immature (IgM^-) and mature (IgM^+) B-lineage cells (22). Recent data suggest that B-cell progenitors expressing B220 and low levels of Thy-1 ($B220^+$ $Thy\text{-}1^{lo}$) give rise to phenotypically distinct pre-B cells ($B220^+$ $Thy\text{-}1^-$) (23). Thus, multiparameter FACS analysis using fluorochrome-conjugated monoclonal antibodies specific for B220, Thy-1.2, and IgM allowed us to identify and distinguish $B220^+$ $Thy\text{-}1.2^{lo}$ IgM^- and $B220^+$ $Thy\text{-}1.2^-$ IgM^- cells, as well as more mature $B220^+$ $Thy\text{-}1.2^-$ IgM^+ cells.

A low percentage (≈4%) of $B220^+$ IgM^- cells was detected in bone marrow of nontransgenic scid mice, whereas the frequency of these cells in μ-transgenic scid mice (≈11%) was comparable to that observed in heterozygous scid mice with or without the μ transgene (Fig. 3). In addition, the majority of $B220^+$ IgM^- cells in μ-transgenic scid mice were qualitatively distinct from the $B220^+$ IgM^- cells in nontransgenic scid mice. That is, multiparameter FACS analysis of bone marrow cells stained with anti-B220, anti-IgM, and anti-Thy-1.2 antibodies revealed that most, if not all, $B220^+$ IgM^- cells in nontransgenic scid mice expressed low levels of Thy-1.2 on their surface, while only ≈35% of $B220^+$ IgM^- cells from μ-transgenic scid and control heterozygous scid mice exhibited this phenotype (Table 1). Thus, ≈65% of the $B220^+$ IgM^- cells in μ-transgenic scid mice are $Thy\text{-}1.2^-$ and presumably correspond to μ-containing pre-B cells. In support of this inference, we have found by immunofluorescence staining that ≈50% of FACS-sorted $B220^+$ bone marrow cells from μ-transgenic scid mice stain for cytoplasmic μ chains; no such cells have been observed in the $B220^+$ fraction isolated from the bone marrow of nontransgenic scid littermates (data not shown).

B cells ($B220^+$ IgM^+) were not observed in the bone marrow of μ-transgenic scid mice, in keeping with the absence of detectable κ chains in this tissue (Fig. 2*A*); such cells did appear in the bone marrow of $\mu\kappa$-transgenic scid mice, though their frequency (3%) was 2- to 3-fold less than that observed in scid heterozygotes with or without the $\mu\kappa$ transgene (Fig. 3). The use of monoclonal antibodies specific for the IgM allotypes Igh-6a and Igh-6b enabled us to identify B cells expressing transgenic μ chains alone ($Igh\text{-}6a^+6b^-$), endogenous μ chains alone ($Igh\text{-}6a^-6b^+$), or both transgenic and endogenous μ chains ($Igh\text{-}6a^+6b^+$). Representative Igh-6a vs. Igh-6b profiles of gated $B220^+$ cells from bone marrow and spleen of $\mu\kappa$-transgenic scid mice and littermate controls are shown in Fig. 4. The majority (≈75%) of splenic B cells from control transgenic heterozygous scid mice were $Igh\text{-}6a^+6b^+$, whereas in the bone marrow, almost all of the detectable B cells (>95%) were $Igh\text{-}6a^+6b^-$. In $\mu\kappa$-transgenic scid mice, all of the detectable B cells in both bone marrow and spleen were $Igh\text{-}6a^+6b^-$ (we have not observed these mice to express endogenous μ chains). However, the number of $Igh\text{-}6a^+6b^-$ splenic B cells was extremely low and represented <1% of the cells analyzed (Fig. 4 *Bottom right*; the

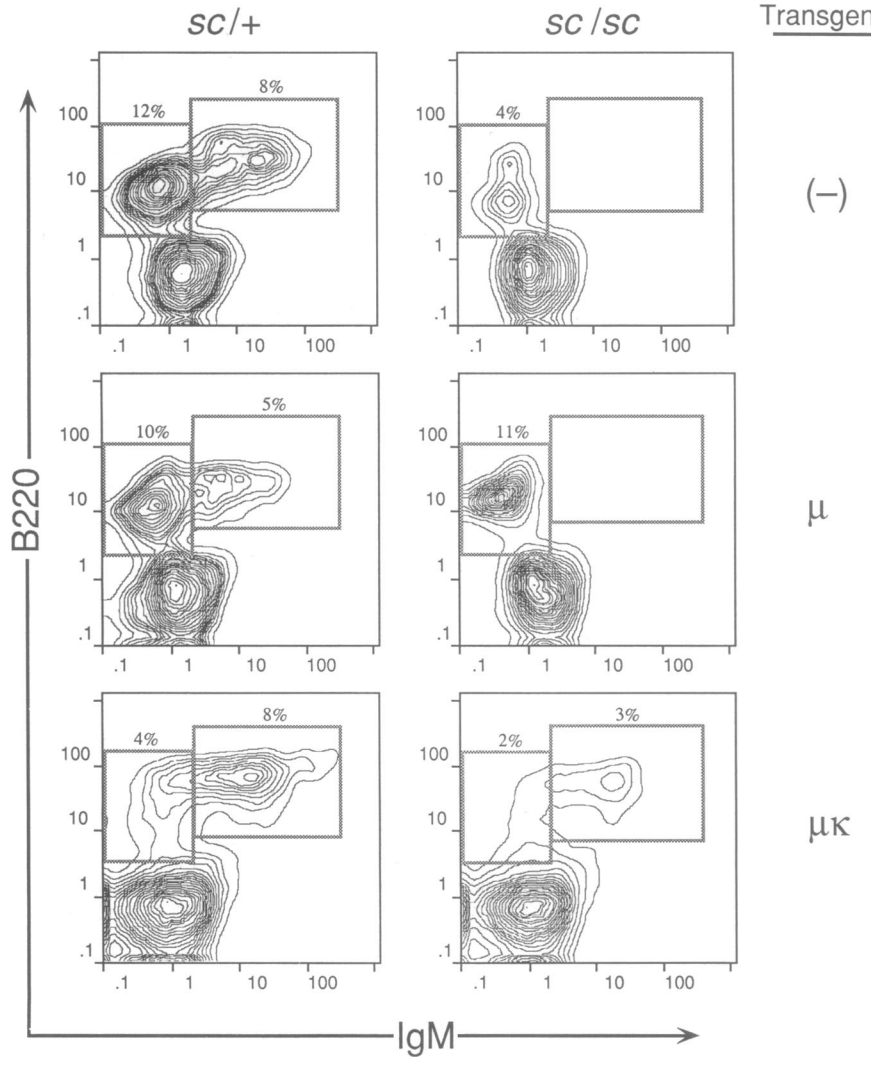

FIG. 3. Demonstration of newly arising B-lineage cells in the bone marrow of μ- and $\mu\kappa$-transgenic scid mice by FACS analysis. Seven mice of each genotype were examined individually. The contour plots in each panel are representative of the surface phenotypes of B-lineage cells in bone marrow of 3-month-old littermates that were homozygous or heterozygous for *scid* (i.e., *sc/sc* or *sc/+*) and with or without (−) the μ or $\mu\kappa$ transgenes. The boxes within each panel delineate less mature ($B220^+$ IgM^-, left) and more mature ($B220^+$ IgM^+, right) cells whose frequencies are given as percentage of total nucleated cells (100,000 cells per analysis). Cells were stained with allophycocyanin/anti-B220 (RA3-6B2; ref. 24) and fluorescein/anti-IgM (331.12; ref. 25).

Table 1. Frequency of immature B-lineage cells in the bone marrow of *scid/scid* and *scid/+* littermates with or without the μ transgene (*Tg*)

Mouse genotype	Frequency, %	
	$B220^+$ Thy-1.2^{lo} IgM^-	$B220^+$ Thy-1.2^- IgM^-
+/+, *scid/scid*	3.3 ± 0.4	0.3 ± 0.2
Tg/+, *scid/scid*	3.6 ± 0.3	5.7 ± 0.9
+/+, *scid*/+	2.6 ± 0.4	5.5 ± 1.6
Tg/+, *scid*/+	2.6 ± 0.3	6.3 ± 0.6

Percentages of immature B-lineage populations of the indicated phenotypes were obtained by integration of FACS analyses and represent the average values (± SEM) of four to seven mice examined from each genotype. Reagents employed for cell staining were phycoerythrin/anti-B220 (RA3-6B2; ref. 24), fluorescein/anti-Thy-1.2 (30H12; ref. 26) and allophycocyanin/anti-IgM (331.12; ref. 25). One hundred thousand cells were examined per analysis.

contours along the diagonal represent nonspecifically stained and/or autofluorescent cells, as the same pattern was observed in the spleen of nontransgenic scid mice). No B cells were detectable in the lymph nodes or peritoneal cavity of $\mu\kappa$-transgenic scid mice (data not shown).

DISCUSSION

We have shown that introduction of a functionally rearranged μ gene into the genome of scid mice enables early B-cell progenitors in the bone marrow to synthesize cytoplasmic μ chains and differentiate into pre-B cells. Whether these pre-B cells attempt Ig light-chain gene rearrangements is not yet clear. If such rearrangements occur, they presumably would be aberrant as a result of the *scid* defect and thus preclude further differentiation. Indeed, the apparent differentiation of scid pre-B cells into B cells ($B220^+$ IgM^+) required the introduction of both a functional μ and a functional κ gene into the scid mouse germ line. This stepwise removal of the developmental arrest in the differentiation of lineage-committed scid B cells is consistent with the hypothesis that *scid* impairs VDJ recombination in developing lymphocytes. Also consistent with this idea is the recent report of Scott *et al.* (29) showing the presence of mature T cells in the thymus of transgenic scid mice containing a functional α and β T-cell receptor gene.

What is puzzling about our results is that virtually all of the detectable B cells in $\mu\kappa$-transgenic scid mice ($B220^+$ Igh-$6a^+6b^-$) resided in the bone marrow, whereas in $\mu\kappa$-transgenic scid heterozygotes such cells were also found in the spleen and represented ≈25% of all detectable splenic B cells (see Fig. 4). To test whether the absence of functional T cells could have delayed or abrogated the development of peripheral B cells in $\mu\kappa$-transgenic scid mice, we injected some of these mice with thymocytes or bone marrow cells from normal C.B-17+/+ mice. Although this resulted in the reproducible appearance of $CD3^+$ T cells and Igh-$6a^-6b^+$ B cells in the case of bone marrow transfer, no detectable increase in $B220^+$ Igh-$6a^+6b^-$ host cells was observed in the

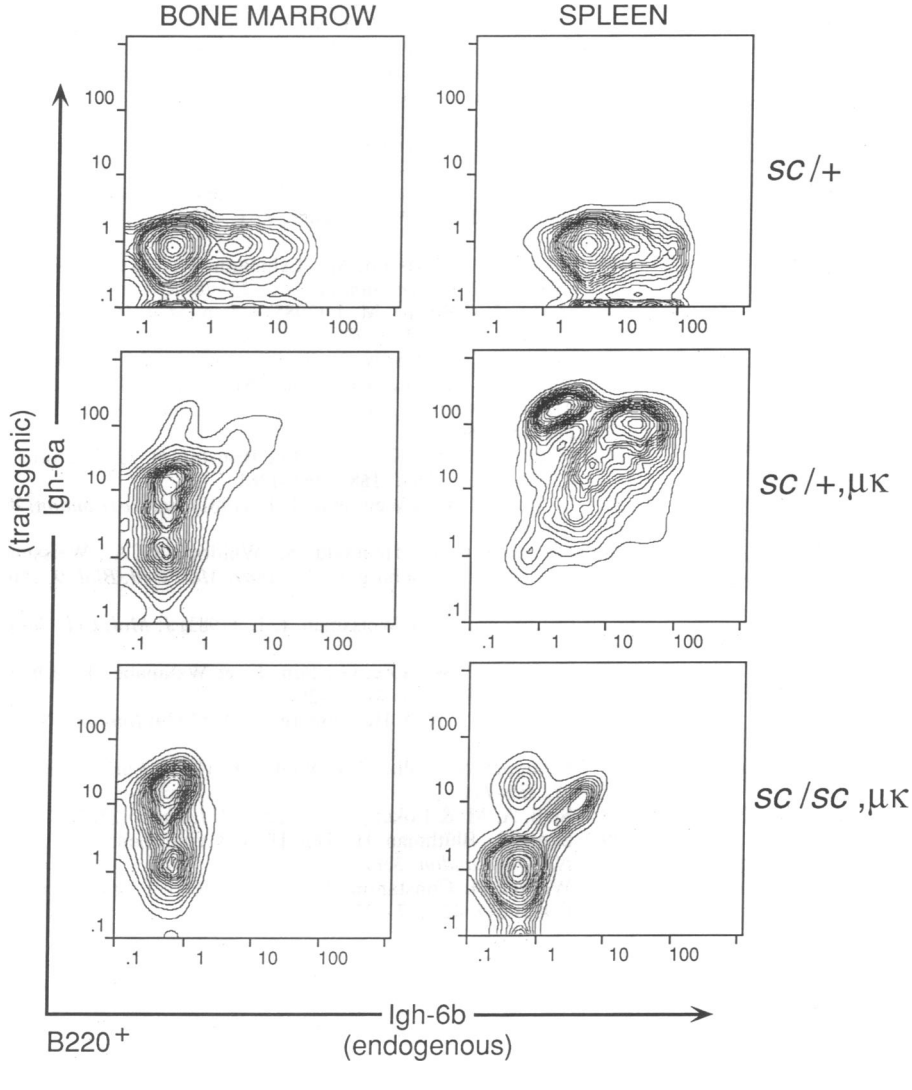

FIG. 4. IgM^+ B cells expressing transgene-encoded μ chains develop in the bone marrow of $\mu\kappa$-transgenic scid mice but fail to populate the spleen. Shown are Igh-6a versus Igh-6b profiles of gated $B220^+$ cells. Four mice of each genotype were examined individually. The contour plots in each panel are representative of the surface phenotypes of B-lineage cells in bone marrow and spleen of 3-month-old littermates that are homozygous or heterozygous for *scid* (i.e., *sc/sc* or *sc/+*) and with or without the $\mu\kappa$ transgene. The frequencies of IgM^+ cells in each panel shown are as follows: in the bone marrow, 8% in *sc/+* mice (all Igh-$6b^+$), 8% in *sc/+*, $\mu\kappa$ mice (7% Igh-$6a^+6b^-$ cells and <1% Igh-$6a^+6b^+$), and 3% in *sc/sc*, $\mu\kappa$ mice (all Igh-$6a^+$); and in the spleen, 40% in *sc/+* mice (all Igh-$6b^+$), 20% in *sc/+*, $\mu\kappa$ mice (5% Igh-$6a^+6b^-$ cells and 15% Igh-$6a^+6b^+$), and 0.24% in *sc/sc*, $\mu\kappa$ mice (all Igh-$6a^+6b^-$; contours along the diagonal represent nonspecifically stained and/or highly autofluorescent cells). Bone marrow and spleen cells were stained with monoclonal antibodies specific for B220 (allophycocyanin/RA3-6B2), the transgene μ-chain allotype (phycoerythrin/anti-Igh-6a, RS-3.1; ref. 27), or the endogenous μ-chain allotype (fluorescein/anti-Igh-6b, AF6-78.25; ref. 28).

spleen and the mice remained serum Igh-6a$^-$ (unpublished results).

How then can we explain the very low numbers of transgene-expressing B cells in the peripheral lymphoid tissues of $\mu\kappa$-transgenic scid mice? One possible explanation is that lineage-committed scid B cells attempt rearrangement of their endogenous Ig genes despite the presence of a functional $\mu\kappa$ transgene. It is evident from Fig. 4 that endogenous rearrangements were not excluded, since $\mu\kappa$-transgenic heterozygous scid mice expressed both transgenic and endogenous μ chains in the majority of their splenic B cells. Rearrangement of endogenous κ-chain genes was also clearly evident from the prominent expression of endogenous κ chains in the spleen lysates of $\mu\kappa$-transgenic heterozygous scid mice (Fig. 2*B*); endogenous κ chains were also detected in bone marrow lysates of these mice. Rearrangement of endogenous Ig genes presumably would be lethal to most developing B-lineage cells in $\mu\kappa$-transgenic scid mice, as it would result in a high frequency of chromosomal breaks due to the inability of the scid VDJ recombinase system to join V, D, and J coding elements (11, 12). Accordingly, the few B cells found in the spleen of $\mu\kappa$-transgenic scid mice may have resolved their broken chromosome ends by some non-VDJ recombination mechanism, or the $\mu\kappa$ transgene may have completely excluded endogenous Ig gene rearrangements in these particular cells. Thus, this explanation predicts that peripheral transgene-expressing B cells would readily develop in scid mice with μ and κ transgenes that completely suppress rearrangement of endogenous Ig genes.

Incomplete allelic exclusion of endogenous Ig loci by the $\mu\kappa$ transgene may also explain why the number of Igh-6a$^+$6b$^-$ cells was 2- to 3-fold less in the bone marrow of $\mu\kappa$-transgenic scid mice than in $\mu\kappa$-transgenic scid heterozygotes. If expression of the $\mu\kappa$ transgene in developing B-lineage cells precedes or is contemporaneous with the initiation of endogenous D to J rearrangements at the Ig heavy-chain locus, then Igh-6a$^+$6b$^-$ expressing B lineage cells would appear early in the lymphopoietic bone marrow. However, in the case of $\mu\kappa$-transgenic scid mice, most of these cells would die prematurely due to chromosomal breaks resulting from attempted rearrangement of endogenous D and J elements. Interestingly, in μ-transgenic scid bone marrow the frequency of early B-lineage cells (B220$^+$ IgM$^-$) appeared normal (see Fig. 3). This may reflect more complete suppression of endogenous D to J rearrangements by the μ transgene (30) than by the $\mu\kappa$ transgene.

Notwithstanding the preceding considerations, we cannot exclude the possibility that *scid* affects more than one process in lymphocyte differentiation including VDJ recombination—e.g., a process occurring between Ig heavy- and light-chain gene recombination such that normal numbers of B cells fail to develop in $\mu\kappa$-transgenic scid mice. Resolution of this issue will depend on demonstrating the induction of Ig light-chain gene rearrangement in developing B-lineage cells of μ-transgenic scid mice and defining the conditions necessary for the generation of functional peripheral B cells in $\mu\kappa$-transgenic scid mice.

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